**Isolation and Characterization of plant TAF9, an
Orthologous Gene for TATA Binding Protein Associated
Factor 9, from Wintersweet (Chimonanthus praecox)**

Journal:	<i>Canadian Journal of Plant Science</i>
Manuscript ID	CJPS-2016-0403.R1
Manuscript Type:	Article
Date Submitted by the Author:	16-Apr-2017
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Keywords:	general transcription factor, TAF9 gene, expression pattern, Arabidopsis thaliana

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Isolation and Characterization of plant *TAF9*, an Orthologous Gene for TATA Binding Protein Associated Factor 9, from Wintersweet (*Chimonanthus praecox*)

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Abstract

TAF9 is one of the TATA-binding protein-associated factors (TAFs) that constitute the TFIID complex. We isolated a plant TAF9 ortholog from the *Chimonanthus praecox* cDNA library, and named it CpTAF9. The CpTAF9 protein contained the conserved TAF9 and H2A superfamily domain, which is highly conserved among the TAF9s of other organisms. It also showed a specific tissue expression pattern that the transcript level of *CpTAF9* was higher in mature leaves than in other tissues. In addition, the *CpTAF9* expression in wintersweet leaves could be induced by treatment with salt or high-temperature, or by exogenous abscisic acid application. Overexpression of *CpTAF9* in *Arabidopsis* under the cauliflower mosaic virus 35S promoter improved salt and high-temperature stress tolerance to some extent. These results demonstrated that *CpTAF9* may have a role in gene regulation associated with salt and heat stress responses in wintersweet, providing a foundation for further elucidating the molecular mechanisms that underlined TAF-mediated control of the stress response processes in plant.

Key words: general transcription factor, TAF9 gene, expression pattern, *Arabidopsis thaliana*

INTRODUCTION

Strict regulation of gene expression happens at the level of transcription. RNA polymerase II (RNAP II) initiates transcription, and several general transcription factor (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH) combinations are required for synthesis of pre-mRNA by RNAP II (Thomas and Chiang, 2006; Juven-Gershon and Kadonaga, 2010). The core promoter-recognition complex, TFIID, is in the transcription of protein-coding genes in eukaryotes. TFIID is facilitated to bind to different core promoter elements by the TAF proteins. In addition, TAFs can also be used as co-activators to regulate basal transcription machinery activity with specific transcription factors (Burley and Roeder, 1996; Cler et al., 2009).

Over the last two decades, TAFs have been extensively studied. TAFs from *S. cerevisiae*, *S. pombe*, *C. elegans*, *D. melanogaster*, and *H. sapiens* have been assessed and strong conservation has been shown in their amino acid sequences (Tora, 2002). In plants, the TAF genes were identified from model plants, such as *Arabidopsis thaliana* (Lago et al., 2004; Lawit et al., 2007), wheat (Kawata et al., 1992) and rice (Zhu et al., 2002). AtTAF1 functions as a coactivator that integrates light signals and histone acetylation to initiate light-induced gene transcription (Bertrand et al., 2005). TAF proteins in wheat are co-activators and have been shown to have a protein size ranging from approximately 30 to 250 kDa (Washburn et al., 1997). Several TAFs have been shown to have expression patterns that are developmental and/or tissue stage-specific, and they are necessary for only a subset of gene expressions (Hiller et al., 2001; Moraga and Aquea, 2015). For example, the *AtTAF6* gene is expressed in

different tissues in *Arabidopsis*, and the mutant *AtTAF6* gene affects pollen tube growth specifically. Also, overexpression of *AtTAF10* in *Arabidopsis* improved seed germination rates under osmotic stress (Gao et al., 2006), and was also found to yield mostly vascular tissue preferential expression (Tamada et al., 2007). Similarly, the *FtTAF10* gene from *Flaveria trinervia* was shown to regulate expression of some genes in vascular tissues (Furumoto et al., 2005). Moreover, *AtTAF12* is required for ethylene response in *Arabidopsis* (Robles et al., 2007), and it was also found that the *AtTAF12* protein regulates correlative genes that participate in late signaling processes that govern cytokinin responses, including cell proliferation and differentiation (Kubo et al., 2011). Genetic approaches also showed that the *AtTAF5* gene has a critical role in regulating pollen tube growth and male gametogenesis. Also, it is necessary for molecular mechanisms that regulate indeterminate inflorescence meristems (Mougiou et al., 2012). Moreover, *TAF13* has been characterized from *Arabidopsis*, and results suggested that *TAF13* functions together with the *Polycomb* Repressive Complex 2 (PRC2) in transcriptional regulation during seed development (Lindner et al., 2013).

In these TAFs proteins, *TAF9*, a TATA-binding protein associated factor (TAF), is conserved from yeast to humans, and it shared by SAGA (Spt-Ada-Gcn5 acetyl transferase) and TFIID, two transcription coactivator complexes. *TAF9* has been identified in some species, such as humans (formerly called hTAF_{II}31 or hTAF_{II}32 [Klemm et al., 1995; Lu and Levine, 1995]), *Drosophila melanogaster* (formerly dTAF_{II}40 [Thut et al., 1995]), and yeast (formerly yTaf17p [Moqtaderi et

al., 1996]). In *Saccharomyces cerevisiae*, genome-wide expression analysis indicated that depletion of TAF9 causes a decrease of about 60% to 65% of gene expression at a transcriptional level (Moqtaderi et al., 1998; Shen et al., 2003). Moreover, it showed that TAF9 may be involved in the regulation of genes associated with apoptosis. In humans, TAF9 and TAF9B are involved in transcriptional activation, and also repressed sets of genes that are distinct but overlapping (Frontini et al., 2005).

However, in plants, only a limited number of TAFs have been identified, and the functions of most TAFs in plants are yet to be determined. In this study, we isolated a TAF9 cDNA from *Chimonanthus praecox* encoding a protein that is highly homologous to TATA Binding Protein Associated Factor 9, and identified its tissue-specific and induction expression pattern. Moreover, the overexpression of CpTAF9 in *Arabidopsis* showed improving stress tolerance in transgenic *Arabidopsis*.

MATERIALS AND METHODS

Plant Materials

Wintersweet [*Chimonanthus praecox* (L.) Link] plants used in our study were grown in the nursery of Southwest University, Chongqing, China. Different tissues, including root, stem, leaves and flowers, were collected from the wintersweet plants. All samples were immediately frozen in liquid nitrogen and then the samples were stored in a freezer at -80°C . For ectopic analysis, we used *Arabidopsis* (Columbia ecotype), which was stored in the Chongqing Engineering Research Center for Floriculture, Southwest University.

RNA Extraction

An RNAPrep pure Plant RNA Purification Kit (Tiangen Biotech, Beijing, China) was used to extract total RNA from samples. The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA) was used to assess the quality and quantity of total RNA, and then the total RNA was also visualized with 1% agarose gel electrophoresis.

Cloning of The Full-length CpTAF9 cDNA

A PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan) was used to synthesize first-strand cDNA from total RNA (1 µg). A randomly expressed sequence tag (EST) sequence screened from the wintersweet cDNA library (Sui et al., 2012), and the full-length gene *cDNA* was isolated with a rapid amplification of cDNA ends (RACE) technique by using primer P1, as shown in Table 1. The following program was used for PCR analyses: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 72 °C for 60 s, and 72 °C for 10 min extension. The PCR product was separated with a 1.2% agarose gel and purified with an Agarose Gel DNA Extraction Kit (TaKaRa Bio). The fragment was inserted into a pMD18-T vector (TaKaRa Bio) and sequenced by Invitrogen Co., Ltd., Shanghai, China. This cDNA comprised 842 base pairs, and the deduced amino acid sequence showed a high similarity to TAF9 protein. This gene was named *CpTAF9*.

Bioinformatics Analysis

The National Center for Biotechnology Information (NCBI) protein-BLAST program was used to search for sequence homology. To predict the isoelectric point and molecular weight, ExPASy software (Swiss Institute of Bioinformatics, 2005) was

used. The cell localization of the protein was predicted based on its amino acid sequence using PSORT software (Nakai, 2007). Multiple sequence alignment was determined using BioEdit software. The neighbor-joining (NJ) method of MEGA software, version 4.0 (Tamura et al., 2007) was used to create a phylogenetic tree.

Real-time Polymerase Chain Reaction Assessment

A PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan) was used to reverse transcribe equal amounts of DNA-free RNA (5 mg) from different tissues into cDNA. Real-time polymerase chain reaction (PCR) was carried out according to Ma et al, 2012. The relative transcript abundance was calculated with the genes for tubulin and actin (Sui et al., 2012). Gel electrophoresis was used to confirm the sizes of the amplified products. Negative controls without templates were concurrently used. There were three biological replicate runs for all quantitative PCRs and there were three technical replicates per experiment. Gene-specific primers for expression analysis are listed in Table 1.

Vector Construction and Arabidopsis Transformation

The coding region of *CpTAF9* was amplified using the primer P3 (Table 1). The digested PCR product was ligated into the pCambia1300 vector between the corresponding restriction sites under the control of a CaMV 35S promoter. The recombinant plasmid was sequenced to confirm the correct insertion and electroporated into cells of *Agrobacterium tumefaciens* strain GV3101s. The genetic transformation of wild-type *Arabidopsis* plants was determined using a floral-dip method (Clough and Bent, 1998). The seeds of transgenic plants were screened on

solid half-strength Murashige-Skoog medium (MS) containing 50 $\mu\text{g L}^{-1}$ hygromycin. T_1 transgenic plants were confirmed via real-time PCR using CpTAF9 primers, as shown in Table 1. T_3 plants were used for the stress tolerance experiments.

Heat and Salt Treatments of Transgenic Arabidopsis Plants

For germination analysis, the seeds of wild-type (WT) and T_3 transgenic *Arabidopsis* (OE1 and OE2) were sown on MS medium containing 0, 50, 100 or 150 mM NaCl, respectively. The germination percentage was determined at the indicated time. To measure root growth, the *Arabidopsis* seeds were germinated on MS agar for 7 days, and the seedlings were transferred to fresh medium containing 0, 50, 100 and 150 mM NaCl. The plates were positioned vertically on shelves to assist the evaluation of root growth. For high-temperature stress, 3-week-old seedlings were exposed to a temperature of 42 °C for 4 h or 6 h and then returned to normal conditions. All experiments were performed three times independently.

Statistical Analysis

Statistical analysis was conducted using SPSS software. The significance of differences between the control and transgenic *Arabidopsis* plants was analyzed with a Student's *t*-test. It was considered to be significant with a P-value of 0.05.

RESULTS

Identification and Phylogenetic Analysis of CpTAF9

The cDNA library of wintersweet was used to isolate a novel gene, designated CpTAF9, by random clone selection and sequencing. The full length cDNA comprised 842 base pairs and contained an open reading frame (ORF) of 603 bp, a 5'-UTR (5'

untranslated region) of 57 bp and a 3'-UTR of 182 bp. Using ExPASy software, the theoretical isoelectric point and molecular weight of the deduced CpTAF9 protein were predicted to be 5.214 kDa and 22.453 kDa, respectively. According to the predicted localization by PSORT, CpTAF9 protein may be located in the nucleus.

CpTAF9 sequence homology was verified using the BLAST algorithm on the NCBI server. The results revealed that CpTAF9 protein exhibits a high sequence identity with the TAF9-like proteins from other plant species such as *Populus trichocarpa* (79%, XP_002299587), *Ricinus communis* (78%, XP_002521322), *Glycine max* (75%, NP_001236385) and *Vitis vinifera* (75%, XP_002273931). Furthermore, a multiple sequence alignment was performed, comparing CpTAF9 protein with proteins homologous to Arabidopsis (*Arabidopsis thaliana*), grape (*Vitis vinifera* L.), cucumber (*Cucumis sativus*), corn (*Zea mays* L.) and tomato (*Solanum lycopersicum*), using BioEdit software (Fig. 1). Alignment analysis revealed that CpTAF9 protein contained the same conserved TAF9 and H2A superfamily domain as other homologous proteins. According to these results, we concluded that the predicted protein was the TAF9 protein of wintersweet, and that the encoding gene belonged to the TAF gene family.

To investigate the phylogenetic relationship between CpTAF9 and other TAF9-like proteins, a phylogenetic tree was constructed by the NJ method with MEGA software, version 5.0. The phylogenetic tree included three groups, as shown in Figure 2. CpTAF9 was within a dicotyledon group cluster, and was closest to *Prunus mume*.

Expression Pattern of *CpTAF9* in Wintersweet

The *CpTAF9* gene showed different levels of expression levels in different wintersweet tissues (Fig. 3). Among all the six tested tissues, *CpTAF9* was most highly expressed in the mature leaves, with lower expression in young leaves, flowers and cotyledons. To better understand the function of the *CpTAF9* gene, we investigated *CpTAF9* transcript levels in the leaves of 2-month-old wintersweet seedlings, which had been subjected to exogenous 100 μ M ABA, or abiotic stresses such as salt stress (1 mol/L NaCl), low temperature (4 $^{\circ}$ C) or high temperature (42 $^{\circ}$ C). Real-time PCR analysis indicated that *CpTAF9* gene expression in wintersweet leaves could be induced by exogenous ABA application, salt stress or high-temperature treatment, as shown in Figure 4. In response to salt stress, *CpTAF9* transcripts accumulated rapidly, with an eight-fold change at 15 min after initiation of salt treatment. Moreover, *CpTAF9* expression remained at a high level 1 h, 6 h and 12 h after salt treatment. *CpTAF9* mRNA abundance peaked with a 10-fold change over the untreated level after 6 h of high-temperature treatment; the expression level subsequently decreased at 12 h. After ABA treatment, *CpTAF9* mRNA transcripts exhibited a four-fold increase at 12 h, and reached the highest level 24 h after exogenous ABA treatment. These results suggested that *CpTAF9* may be involved in responses to abiotic stress in wintersweet.

Salt and Heat Tolerance of Transgenic Arabidopsis

We developed transgenic Arabidopsis plants in which *CpTAF9* was overexpressed under the CaMV 35S promoter to investigate the biological function of the *CpTAF9*

gene in plants. In addition, two T3 transgenic lines (OE1 and OE2) overexpressing *CpTAF9* were selected to assess how *CpTAF9* affects salt or heat tolerance.

To explore the involvement of *CpTAF9* in salt stress, we measured the germination rate in transgenic and wild-type *Arabidopsis* under salt stress conditions. A greater than 99% germination rate was recorded for seeds sown on control MS medium. When exposed to 100 mM NaCl for 7 days, the germination rates of transgenic seeds were 78% and 70%, which were significantly higher than the germination rate of the WT seeds (39.6%) under salt treatments. We also compared the root growth between transgenic and WT seedlings under salt stress. The length of main and lateral root in transgenic seedlings was significantly longer than that of WT at 100 and 150 mM NaCl (Fig. 5).

We further investigated the biological function of *CpTAF9* gene in heat stress. As shown in Figure 6, when exposed to high temperature (42 °C), the malondialdehyde (MDA) content of transgenic *Arabidopsis* was significantly lower than that of the wild-type seedlings. Moreover, the change in cell membrane permeability of transgenic *Arabidopsis* was also obviously lower than that in wild-type seedlings at 4 h and 6 h after high-temperature treatment (Fig.6). These results suggested that the overexpression *CpTAF9* in transgenic *Arabidopsis* could increase tolerance to salt and heat stress.

DISCUSSION

The first step in gene expression is transcription. It can be regulated to guarantee

suitable levels of the gene product. It is critical to understand which factors work together to achieve the correct regulation of transcription to understand the mechanism of gene expression and further affect the development and function in organisms (Zaborowska et al., 2012). In plants, the research focused on the functional analysis of transcription factors that may be involved in regulation of different processes, such as biotic and abiotic stress responses, metabolic pathways, and development, which have been intensively studied. However, studies on the basal transcription machinery, which is important for the initiation of transcription in plants, lag far behind the intensive research on general transcription factors in mammals, flies and yeast (Albright and Tjian, 2000; Srivastava et al., 2015).

So far, several isolation and functional analyses have been performed for TAFs in plants, and the results indicated that plant TAFs may play important roles in activating transcription and in specific regulation of distinct subsets of gene expression (Moraga and Aquea, 2015). Here, we reported the isolation of a *TAF9* gene from *Chimonanthus praecox*. Based on its amino acid sequence, it is showed high similarity to the sequence of other plant TAF9s (Fig. 1). We suggested that CpTAF9 may be a plant TAF9 ortholog, and the plant TAF9s showed a high level of sequence conservation. In addition, our analysis showed that the CpTAF9 protein may be located in the nucleus, which concords with its putative function as a transcriptional regulator. Moreover, the *CpTAF9* expression analysis showed that *CpTAF9* was expressed in all tissues. In *Arabidopsis*, the RT-PCR experiment showed that the TAF genes were expressed in roots, rosette leaves and inflorescences (Lago et

al., 2004). These expression patterns of TAF genes are consistent with their putative role in the basal transcription apparatus. Meanwhile, the *CpTAF9* also showed a specific tissue expression pattern in that the transcript level of *CpTAF9* was higher in mature leaves than in other tissues. In addition, *CpTAF9* expression in wintersweet leaves could be induced by exogenous ABA application, or by salt or high-temperature treatment. Exogenous auxin and cytokinin treatment was used to up-regulate the expression of *AtTAF10*. The results suggested that *AtTAF10* might play a role in cytokinin and/or auxin signal transduction pathways for morphogenesis (Tamada et al., 2007). The induced expression pattern of *CpTAF9* indicated that the *CpTAF9* may play a role in responses to abiotic stress, such as salt or heat stress, in wintersweet.

Environmental stresses often cause molecular level and physiological changes in plants. Salinity may reduce germination percentage due to higher osmotic pressures. In our study, the germination rates of transgenic seeds were significantly higher than that the germination rate of the WT seeds when exposed to 100 mM NaCl for 7 days. Moreover, the *CpTAF9* transgenic seedlings had longer main and lateral roots under salt stress. It suggested that the salt sensitivity was reduced at some extent during seed germination and growth by overexpressing of *CpTAF9* gene in *Arabidopsis*. Heat stress influences photosynthesis, cellular and subcellular membrane components, protein content in cell, and antioxidant enzyme activity. Heat stress also induces oxidative stress in plants caused by the generation and the accumulation of reactive oxygen species (ROS), such as super-oxides (O_2^-), hydrogen peroxide (H_2O_2). Our

results showed that MDA content and change in cell membrane permeability of the transgenic *Arabidopsis* were both significantly lower than those of the wild-type seedlings during high-temperature treatment. It suggested that the enhanced capacity of transgenic plants for scavenging ROS than the WT. These results suggested that *CpTAF9* may be involved in responses to salt and heat stress in wintersweet. A TBP-Associate factor, AtTAF10 was reported that overexpression of *AtTAF10* in *Arabidopsis* improves seed tolerance to salt stress during germination and the knock-down mutant is more sensitive to salt stress. And the results indicated that the transcription initiation factor as a physiological target of salt toxicity in plants (Gao et al., 2006). Plants have been shown to respond to abiotic stress by changing their gene expression pattern. This then regulates a series of biochemical processes to reduce stress damage. Also, a hypothesis was developed suggesting that a disturbance in the balance of the members of transcriptional mediator complexes, SAGA and/or TFIID produce an effect on gene expression in regard to development progression and environmental stress responses in plants (Furumota et al., 2005). *CpTAF9* overexpression has an effect on gene expression profiles that relate to salt and heat stress, and this still needs to be elucidated.

In conclusion, the isolation and characterization of the *CpTAF9* gene presented here is only part of functional analysis of these TAFs in plants. The molecular mechanism underlying TAF-mediated control of the stress response processes in plants needs intensive study in the future.

FUNDING

This research is funded by the National Natural Science Foundation of China (grant no. 31370698), Chongqing Research Program of Basic Research and Frontier Technology (No. cstc2016jcyjA0153), the Fundamental Research Funds for the Central Universities (XDJK2016C187 and XDJK2015B013).

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Table 1. Primer sequences used in this study for polymerase chain reaction analysis in wintersweet and *Arabidopsis*

No.	Name	Oligonucleotides(5'-3')	Note
P1	<i>CpTAF9-F</i>	GTGTTGGTACCCGGAAT	For full-length cloning of <i>CpTAF9</i> from
	<i>CpTAF9-R</i>	GCTCAAAGCTCAAAACCATAAT	wintersweet
P2	<i>CpTAF9-SF</i>	AAGGAAGGAGGAATGGAGGAAG	For qRT-PCR analysis of <i>CpTAF9</i> in
	<i>CpTAF9-SR</i>	TTGATGGACGACACGAGGTT	wintersweet or transgenic <i>Arabidopsis</i>
P3	<i>CpTAF9-VF</i>	CGGGATCCAGATGGCGGACGCAGG	For construction of <i>CpTAF9</i>
		AGGAG	
		CGGAATTCTCCACATTCCAAACC	
P4	<i>CpTAF9-VR</i>	CGACAGT	overexpression vector
		AGGCTAAGATTCAAGACAAGG	
		TTGGTCGCAGCTGATTGCTGTG	
P5	<i>Actin-bF</i>	AGGCTAAGATTCAAGACAAGG	Reference genes for qRT-PCR analysis
	<i>Actin-bR</i>	TTGGTCGCAGCTGATTGCTGTG	in wintersweet
P6	<i>Tublin-F</i>	GTGCATCTCTATCCACATCG	Reference gene for qRT-PCR analysis in
	<i>Tublin-R</i>	CAAGCTTCCTTATGCGATCC	<i>Arabidopsis</i>

Note: The underlined bases in primers are the restriction sites. qRT-PCR, quantitative reverse transcription-polymerase chain reaction

Fig. 2. Phylogenetic relationships of CpTAF9 with other TAF9 amino acid sequences. The phylogenetic tree file was produced using MEGA 4.0 software (Tamura et al., 2007). The bootstrap values were obtained by the neighbor-joining method and indicate the divergence of each branch. The scale indicates the branch length.

Fig. 3. Real-time quantitative polymerase chain reaction (PCR) analyses of transcript levels in different tissues. Data were normalized against a reference of wintersweet actin and tubulin genes. Three biological replicates were used for all quantitative PCRs for each gene, with three technical replicates per experiment; the error bars indicate SD.

Fig. 4. Expression patterns of *CpTAF9* gene from wintersweet in response to various treatments, including salt stress (100 mM NaCl), low-temperature stress (4 °C), high temperature (42 °C) and 100 uM exogenous abscisic acid (ABA). Data were normalized against a reference of wintersweet actin and tubulin genes. Three biological replicates were used for all quantitative PCRs for each gene, with three technical replicates per experiment; the error bars indicate SD.

Fig. 5. Salt stress tolerance analysis of transgenic *Arabidopsis* plants overexpressing CpTAF9. A. Seed germination rate of *CpTAF9* overexpression under salt stress. Transgenic lines OE1, OE2 and WT plants grew on MS medium supplemented with 0, 50, 100 or 150 mM NaCl, respectively, for 15 days after sowing. Three independent experiments were performed. B. Root growth phenotype of transgenic and WT seedlings on medium containing NaCl (0, 50, 100 or 150 mM). C. Main root and lateral root length of transgenic and WT seedling. Asterisks indicate a significant difference from WT ($p < 0.05$).

Fig. 6. Heat stress tolerance analysis of transgenic *Arabidopsis* plants overexpressing CpTAF9. A. The phenotype of transgenic lines OE1, OE2 and WT plants after high temperature treatment. BT, before treatment; AT, after treatment. B. The MDA content of wild-type and transgenic *Arabidopsis* plants under high-temperature stress. C. Cell membrane permeability changes in wild-type and transgenic *Arabidopsis* plants under high-temperature stress. *Asterisks* indicate a significant difference from WT ($p<0.05$).